ATTACHMENT II – PROTOCOL

Ecolab
Study Identification Number 1900080

REGULATED PESTICIDE EFFICACY STUDY PROTOCOL

STUDY TITLE: Synergex Efficacy against Food Contact Surface Associated Biofilm

EPA REG. NO.: 1677-250

ECOLAB GLP STUDY NUMBER: 1900080

PROPOSED STUDY INITIATION/COMPLETION DATES

Initiation	**August 27, 2019**
Completion	**October 18, 2019**

DESCRIPTION OF STUDY OBJECTIVE

Synergex (EPA Registration No. 1677-250) will be tested to determine efficacy against the food contact surface associated biofilms of *Pseudomonas aeruginosa* ATCC 15442 and *Listeria monocytogenes* ATCC 49594 with the test parameters outlined below. EPA approved modifications to U.S. EPA Microbiology Laboratory Branch (MLB) standard operating procedures MB-19; *Growing a Biofilm using the CDC Biofilm Reactor* and MB-20; *Single Tube Method for Determining the Efficacy of Disinfectants against Bacterial Biofilm* will be the test methods utilized in making the food contact surface biofilm efficacy claim.

Test Parameters

Ecolab SOP number:	MS130; *Determination of Antimicrobial Product Efficacy Against Food Contact Surface Associated Biofilm Grown in the CDC Biofilm Reactor Using the Single Tube Test Method*
Test Systems:	*Pseudomonas aeruginosa* ATCC 15442 *Listeria monocytogenes* ATCC 49594
Carrier Type:	304 stainless steel
Number of Carriers:	5 carriers per organism per batch
Exposure Time:	5 minutes
Exposure Temperature:	Room temperature (21±2°C)
Test Substance Batches:	NB14981-47, NB14981-48, NB14981-49
Test Substance Diluent:	500 ppm Synthetic Hard Water
Test Substance Concentration:	The test substance will be diluted at 1.54 oz/3 gallons to result in the active ingredients at or below the lower limits of 469 ppm hydrogen peroxide, 98 ppm peroxyacetic acid and 24 ppm peroxyoctanoic acid

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TEST SUBSTANCE IDENTIFICATION

Test Substance Name: Synergex

Batch Identification: NB14981-47
NB14981-48
NB14981-49

Formula Code: 916834

Date of Manufacture:

Synergex Batch Number	Date of Manufacture
NB14981-47	May 15, 2019
NB14981-48	May 15, 2019
NB14981-49	May 15, 2019

An aliquot of the test substance batches will be retained in the GLP sample storage room at the Ecolab Schuman Campus in Eagan, MN until the quality of the formula no longer affords evaluation. Test substance not dispersed for retention, chemical quality verification or efficacy testing will be stored in Ecolab Microbiological Services cabinet until disposed.

QUALITY ASSURANCE UNIT MONITORING

The protocol, pesticide efficacy in-life and final report are proposed to be inspected by the Ecolab Quality Assurance Unit (QAU) in accordance with their current standard operating procedures. The following proposed Ecolab QA inspections are for planning purposes only and may change. Ecolab QA inspections that are performed, along with their dates and auditors, will be included in the study final report. Changes in Ecolab QA inspections from those proposed below will not require revision of this protocol.

Proposed QAU Monitoring

Protocol Audit
Pesticide Efficacy In-Life Inspection
Final Report Audit

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CHEMICAL QUALITY VERIFICATION

Proposed Experimental Start/Termination Dates

Experimental Start Date: August 2019

Experimental Termination Date: August 2019

Method

The chemical quality verification of the test substance concentrates and test substance use-solution were performed under Ecolab GLP study number 1900071. Chemical analysis was performed on each batch of test substance to determine the concentration of the active ingredients. Chemical analysis was also performed on a single batch of test substance use-solution using batch NB14981-47 diluted in sterile lab purified water at 1.54 oz/3 gallons resulting in a use-solution at or below the lower limits of 469 ppm hydrogen peroxide, 98 ppm peroxyacetic acid and 24 ppm peroxyoctanoic acid.

The chemical quality verification was performed by the Analytical Lab using the methods listed below. The methods have been deemed acceptable by the Analytical Lab and the study sponsor to ensure proper characterization of the test substance concentrate and the test substance use-solution. Statistical treatment of test results may be inherent to the method. Additional volumes and dilutions may be necessary to determine the chemistry of the use-solution sample.

QATM 202B: Hydrogen Peroxide and Peracid Analysis by Titration with Potassium Permanganate

Hydrogen peroxide content is determined by an oxidation-reduction titration with potassium permanganate. After the endpoint of this titration has been reached, an excess of potassium iodide is added to the solution. The potassium iodide reacts with peracids to liberate iodine which is titrated with a standard solution of sodium thiosulfate.

QATM 317: Suppressed Peroxide Titration for Peracids and Hydrogen Peroxide

The method requires two steps for the determination of peracids and hydrogen peroxide. The first step determines the peracid content by iodometric titration while suppressing the hydrogen peroxide oxidative property by dilution and cold temperatures (ice water). The presence of ice in the reaction flask does not interfere with the titration chemistry. This method does not distinguish between various types of peracids; it measures the total content of all peracids present.

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The second step uses the same sample and measures hydrogen peroxide content by the addition of sulfuric acid and molybdenum catalyst. These two reagents rapidly accelerate the oxidation of iodide by hydrogen peroxide. The hydrogen peroxide concentration is determined by subtracting the difference between the volume of titrant used for the peracid endpoint and the volume required to reach the hydrogen peroxide endpoint.

QATM 337: Peroxyacetic Acid and Peroxyoctanoic Acid Determination by Thiosulfate Titration

The method requires two steps for the determination of peroxyacetic acid and peroxyoctanoic acid. The first step determines the POAA content by filtering out POOA and persulfonated oleic acid (PSOA, if present) while suppressing the hydrogen peroxide by cold temperatures. The presence of deionized ice in the reaction flask does not interfere with the titration chemistry.

The second step rinses the POOA and PSOA (if present) off of the filter with solvent. The PSOA is precipitated using calcium acetate and filtered out of the solution. The POOA can then be measured.

QATM 202B was used to determine the hydrogen peroxide concentration in both the concentrates and use-solution. QATM 317 was used to determine the total peracid concentration in the use-solution. QATM 337 was used to determine the peroxyacetic acid and peroxyoctanoic acid concentrations in the concentrates.

The most current QATMs and product specific Bill of Quality will be used during the course of this study for the chemical analysis.

Interpretation of Results

The concentration of the active ingredients in the test substance concentrates will be judged acceptable for pesticide efficacy testing when within the range specified by the Confidential Statement of Formula (CSF) upper and lower certified limits as seen in the table below.

Test Substance Acceptance Limits		
Active Ingredient	**CSF Lower Certified Limit**	**CSF Upper Certified Limit**
Hydrogen Peroxide	9.75%	11.55%
Peroxyacetic Acid	2.04%	2.72%
Peroxyoctanoic Acid	0.49%	0.78%

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The peroxyacetic acid and peroxyoctanoic acid active ingredients could not be distinguished nor analyzed at use solution levels using the validated quality assurance test methods. Instead, the total peracid concentration along with hydrogen peroxide were measured in the use solution to ensure the use solution was prepared at or below the lower limits. Per the sponsor and Primary Validation Report of QATM 317 (Document No.: REPT 317-007-022-P), the lower certified limit of total peracid is 2.43%. After applying the 0.401% (1.54 oz/3 gal) dilution for the use solution, the total peracid lower limit concentration in the use solution is 0.0117%.

The concentration of the active ingredients in the test substance use-solution at the lower limit is <1.0%. Therefore, the lower acceptance limit for these active ingredients will be expanded by 10%. The expanded range is based on 40 CFR § 158.350 (Certified Limits) and was calculated as shown below.

Calculated Lower Acceptance Limit for Hydrogen Peroxide
= [0.0469% - (0.0469 x 0.1)] = 0.04221%

Calculated Lower Acceptance Limit for Total Peracid
= [0.0117% - (0.0117 x 0.1)] = 0.01053%

The calculated upper acceptance limit for the active ingredients in the test substance use-solution were determined by adjusting 2% above the lower limit per U.S. EPA Office of Chemical Safety and Pollution Prevention Product Performance Test Guidelines 810.2000: General Considerations for Testing Public Health Antimicrobial Pesticides – Guidance for Efficacy Testing (February 2018) as shown below.

Calculated Upper Acceptance Limit for Hydrogen Peroxide
= [0.0469% + (0.0469% x 0.02)] = 0.04784%

Calculated Upper Acceptance Limit for Total Peracid
= [0.0117% + (0.0117% x 0.02)] = 0.01193%

The concentration of the active ingredients in the test substance use-solution will be judged acceptable for pesticide efficacy testing if within the expanded acceptance limits listed in the table below.

Test Substance Use-Solution Acceptance Limits	
Active Ingredient	**Expanded Acceptance Limits**
Hydrogen Peroxide	0.04221-0.04784%
Total Peracid	0.01053-0.01193%

Note: For test substances with multiple active ingredients, the test substance is diluted so that all active ingredients are at or below the lower limit. As a result of diluting to ensure all active ingredients present are equal to or less than the lower limit, it is possible that some active ingredients may fall below the lower end of the range. It is acceptable if this occurs.

The Chemical Quality Verification results will be reported in the final report of this study.

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PESTICIDE EFFICACY TESTING

Proposed Experimental Start/Termination Dates

Experimental Start Date	August 2019
Experimental Termination Date	October 2019

Methods

Pesticide efficacy data will be generated by the Microbiology Lab using the most current methods listed below. See the specific methods in the Protocol Appendix.

Method Number	Method Name
MS008	*Synthetic Hard Water Preparation & Standardization*
MS130	*Determination of Antimicrobial Product Efficacy Against Food Contact Surface Associated Biofilm Grown in the CDC Biofilm Reactor Using the Single Tube Test Method*

Test Method Requirement and Test System Justification

The following apply when determining the effectiveness of an antimicrobial product for biofilm efficacy claims on food contact surfaces; 5 test carriers are required on each of three samples, representing different batches, which are to be evaluated on independent test days. The required organisms are *Pseudomonas aeruginosa* (ATCC 15442) and *Listeria monocytogenes* (ATCC 49594). An adaptation of the U.S. EPA MLB SOPs MB-19 and MB-20 for the above stated organisms will be used to determine biofilm efficacy of an antimicrobial product on food contact surfaces. U.S. EPA Office of Chemical Safety and Pollution Prevention Product Performance Guidelines 810.2200 – Disinfectants for Use on Environmental Surfaces – Guidance for Efficacy Testing (February 2018) also applies to this study.

Test Method Justification

Ecolab Microbiological Services SOP MS130; *Determination of Antimicrobial Product Efficacy Against Food Contact Surface Associated Biofilm Grown in the CDC Biofilm Reactor Using the Single Tube Test Method* will be the test method utilized in this study.

Test Systems and Identification

The test systems which will be utilized for this procedure are *Pseudomonas aeruginosa* ATCC 15442 and *Listeria monocytogenes* ATCC 49594. Identifications will be performed by observing the colony morphology and performing Gram stains.

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Organic Soil

No Soil

Statement of Proposed Statistical Method

None

Test Substance Diluent

500 ppm synthetic hard water prepared as described in Ecolab Microbiological Services SOP MS008; *Synthetic hard Water Preparation & Standardization* will be the diluent.

Test Substance Concentration

Antimicrobial efficacy testing will be performed at or below the lower limits of 469 ppm hydrogen peroxide, 98 ppm peroxyacetic acid and 24 ppm peroxyoctanoic acid when diluted at 1.54 oz/3 gallons.

Active Ingredient	CSF Lower Certified Limit	Specific Gravity	Percent Dilution*	Resulting ppm of Active Ingredient
Hydrogen Peroxide	9.75%	1.20	0.401%	469 ppm
Peroxyacetic Acid	2.04%			98 ppm
Peroxyoctanoic Acid	0.49%			24 ppm

*Study proposed dilution: (1.54 oz/3 gallons) x (1 gallon/128 oz) x (100%) = 0.401%

$$\text{Resulting ppm of active ingredient} = \left(\frac{\%Active}{100\%}\right)\left(\frac{\%Dilution}{100\%}\right) \text{x (Specific Gravity) } (10^6)$$

The following calculations will be used to ensure that all active ingredients are at or below their lower limits in the test substance use-solution for use in efficacy testing:

$$\text{Target mass (g) of product} = \frac{(\text{ppm Active at LCL})(\text{Total mass of use-solution})(100\%)}{(10^6)(\%\text{ Active Ingredient Result})}$$

Dilution based on % hydrogen peroxide result from QATM 202B:
g of test substance batch in 500 g to yield 469 ppm hydrogen peroxide (H_2O_2) =

$$\frac{(469\text{ ppm})(500\text{ g})(100\%)}{(10^6)(\%H_2O_2 \text{ in batch})}$$

Dilution based on % peroxyacetic acid result from QATM 337:
g of test substance batch in 500 g to yield 98 ppm peroxyacetic acid (POAA)=

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$$\frac{(98\ ppm)(500g)(100\%)}{(10^6)\ (\%POAA\ in\ batch)}$$

Dilution based on % peroxyoctanoic acid result from QATM 337:
g of test substance batch in 500 g to yield 24 ppm peroxyoctanoic acid (POOA)=

$$\frac{(24\ ppm)(500\ g)(100\%)}{(10^6)\ (\%POOA\ in\ batch)}$$

Dilution procedure for efficacy testing (or equivalent dilution)

Synergex Batch Number	Active Ingredient Concentration[1]	Test Substance Weight*: Diluent Weight*
NB14981-47	Hydrogen Peroxide: 10.80%	2.17 g : 497.83 g
	Peroxyacetic Acid: 2.37%	**2.07 g : 497.93 g**
	Peroxyoctanoic Acid: 0.57%	2.11 g : 497.89 g
NB14981-48	Hydrogen Peroxide: 10.78%	2.18 g : 497.82 g
	Peroxyacetic Acid: 2.32%	2.11 g : 497.89 g
	Peroxyoctanoic Acid: 0.57%	**2.11 g : 497.89 g**
NB14981-49	Hydrogen Peroxide: 10.89%	2.15 g : 497.85 g
	Peroxyacetic Acid: 2.34%	**2.09 g : 497.91 g**
	Peroxyoctanoic Acid: 0.54%	2.22 g : 497.78 g

*Weights may vary by ± 0.03g
The dilution in **bold** will be used for efficacy testing
[1] Active Ingredient Concentration determined under Ecolab GLP Study number 1900071

Test Surface

304 stainless steel coupons (1.27±0.013 cm diameter, approximate 3.0 mm thickness)

Exposure Time/Temperature

The test systems will be exposed to the test substance for 5 minutes at room temperature (21±2°C)

Neutralizer Medium

36 mL D/E Neutralizing Broth

Plating Medium

R2A (*Pseudomonas aeruginosa* ATCC 15442)
BHI (*Listeria monocytogenes* ATCC 49594)

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Incubation Time/Temperature

Test plates will be incubated for 72±4 hours at 35±2°C. Control plates will be incubated for 48±4 hours at 35±2°C.

Test Controls

The following controls will be incorporated with the test procedure for each test system:

a. Control Carrier Enumeration
b. Test Substance Diluent Sterility
c. Neutralization Confirmation
d. Test System Purity
e. Continuous Flow Operation Media Sterility Control

Details on each of the above controls can be found in Ecolab SOP MS130 located in the Protocol Appendix.

Interpretation of Test Results

The performance standard for evaluation of an antimicrobial pesticidal product against biofilm associated with food contact surfaces is ≥6 log reduction in viable *Pseudomonas aeruginosa* biofilm and ≥5 log reduction in viable *Listeria monocytogenes* biofilm from test carriers as compared to the control carriers.

DATA RETENTION

Following the completion of the study, the original final report and raw data will be archived at the Ecolab Schuman Campus in Eagan, Minnesota or at an approved off-site location. All records that would be required to reconstruct the study and demonstrate adherence to the protocol will be maintained for the life of the commercial product plus four years.

TEST SUBSTANCE RETENTION

An aliquot of each batch of test substance will be retained in the GLP sample storage room at the Ecolab Schuman Campus in Eagan, Minnesota until the quality of the formula no longer affords evaluation.

GOOD LABORATORY PRACTICES

This study will be conducted according to Good Laboratory Practices, as stated in 40 CFR Part 160. If it becomes necessary to make changes in the approved protocol, the revisions and reasons for change will be documented, reported to the sponsor and will become part of the permanent file for that study. The sponsor will be notified as soon as it is practical whenever an event occurs that could have an effect on the validity of the study.

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- **Name and Address of Sponsor**

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- **Name and Address of Study Director**

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Sponsor — Date: 8-27-2019

Study Director — Date: 8.27.19

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PROTOCOL APPENDIX

Microbiological Services (MS) Methods:

MS008	*Synthetic Hard Water Preparation & Standardization*
MS130	*Determination of Antimicrobial Product Efficacy Against Food Contact Surface Associated Biofilm Grown in the CDC Biofilm Reactor Using the Single Tube Test Method*
MS102	Attachment